

DR EKKEHARD HEWER (Orcid ID : 0000-0002-9128-0364)

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H3K27me3 immunostaining for sex determination in the context of presumed tissue misidentification

Running title:

H3K27me3 immunostaining for sex determination

Authors:

Joëlle Tschui, Ekkehard Hewer

Institute of Pathology, University of Bern, Bern, Switzerland

Correspondence to:

Ekkehard Hewer, Institute of Pathology, University of Bern, Murtenstrasse 31, 3008 Bern, Switzerland; [ekkehard.hewer@pathology.unibe.ch](mailto:ekkehard.hewer@pathology.unibe.ch); T: +41 31 632 3211; F: +41 31 632 4495

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The authors declare that they have no conflict of interest.

*Sir:* We have read with great interest the correspondence by Dr Crotty<sup>1</sup> and colleagues on the use of XIST chromogenic in situ hybridization for sex determination in the context of presumed tissue misidentification.

The authors refer to H3K27me3 immunostaining which has been reported to label the inactivated X chromosome<sup>2</sup> and therefore may serve the same purpose as XIST ISH, i.e. an in situ analysis of sex. The mentioned study by Dr Schaefer and colleagues beautifully describes the morphological phenomenon and discusses the underlying biology, but does not formally assess the applicability of H3K27me3 immunostaining for sex determination in a routine setting. We have therefore sought to

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close this gap by analysing a variety of neoplastic and non-neoplastic tissues (Table 1) in a blinded fashion. H3K27me3 immunohistochemistry was performed on a Leica Bond III immunostainer with a rabbit monoclonal antibody (clone C36B11; Cell Signalling; at 1:200 dilution with EDTA-based antigen retrieval). Given that in the context of presumed misidentification the tissue fragments of interest are usually small, we included full histological slides and tissue microarrays (TMA; 600  $\mu$ m core diameter) of the same neoplastic and non-neoplastic tissue types.

Sex prediction was correct in all 60 non-neoplastic tissues (full slides and TMA cores), in all 34 TMA glioma samples and in 64 out of 65 full slides from gliomas. The only tumour for which sex was not predicted correctly derived from a female patient and lacked Barr body staining both in tumour cells and non-neoplastic cells. Actual tissue misidentification in this case could be ruled out as microsatellite analysis had been performed in order to test for 1p19q co-deletion.

In sum, H3K27me3 immunohistochemistry predicted sex accurately in 158 out of 159 (99.37%; 95% confidence interval by the modified Wald method: 0.9617 to >0.9999%) which is not significantly different ( $p=1.0000$  by Fisher's exact test) from the results reported by Dr Crotty and colleagues for XIST ISH (268 out of 268; 100; 95% confidence interval: 0.9830 to 1.0000). In the vast majority of samples, sex determination was very straightforward and assessed correctly within seconds. In particular, in many female tissues the presence of Barr body-pattern staining was obvious already at medium-power magnification. In more challenging cases we found the following features useful to distinguish true Barr bodies from non-specific chromatin condensations: Barr bodies were usually single per nucleus, well-defined, predominantly near to the nuclear membrane, easiest to identify in cells which lacked any other non-specific staining and present in a higher proportion of cells (Figure 1).

We conclude that H3K27me3 immunostaining can predict sex with high accuracy in both neoplastic and non-neoplastic tissues. Because of its potential in the diagnosis of malignant peripheral nerve sheath tumour<sup>3,4</sup> it will likely be increasingly used in diagnostic routine across pathology departments in the future.

Easy availability, cost-effectiveness and rapid turnaround lower the threshold to enter a process of tissue identity analysis. They are therefore critical for its widespread use – and thereby ultimately for patient security – in the context of presumed tissue misidentification. We believe that H3K27me3 immunostaining for sex determination fulfils all of these requirements. Furthermore, it offers the advantage of an internal positive control, which may be critical in small tissue fragments of uncertain preservation.

In a broader perspective, we are convinced that a comprehensive approach to tissue misidentification is required: We need to prevent misidentification (i.e. primary prevention in preventive medicine terminology) by appropriate organizational measures at the pre-analytical and analytical level, but also (in the sense of secondary prevention) to have an armamentarium of methods at hand that allows detection of tissue misidentification in a whole variety of scenarios. Such a comprehensive approach will have to include in-situ techniques (of which XIST-ISH and H3K27me3 are promising candidates) to optimally serve patient security.

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JT and EH designed the study and analysed data. EH drafted the manuscript. JT and EH approved the final version of the manuscript.

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## Table

	Male	Female
Normal autopsy tissue – whole slide	19	11
Normal patient tissue – TMA	18	12
Neoplastic – Whole slide (glioma)	39	26
Neoplastic – Tissue microarray (diffuse glioma)	15	19

Table 1. Overview of tissues analysed by H3K27me3 immunostaining for sex determination. Autopsy tissue included heart, lung, thyroid, liver, spleen, brain, skeletal muscle and skin.

## Figure legend

Figure 1. Representative photomicrographs of H3K27me3 stained slides from gliomas of a male (A) and a female (B) patient. Original magnification: 630x

